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Article

Article

The effect of monoamines reuptake inhibitors on aerobic exercise performance in bank voles from a selection experiment

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Abstract

Exercise performance depends on both physiological abilities (e.g., muscle strength) and behavioral characteristics (e.g., motivation). We tested the hypothesis that evolution of increased aerobic exercise performance can be facilitated by evolution of neuropsychological mechanisms responsible for motivation to undertake physical activity. We used a unique model system: lines of bank voles *Myodes glareolus* selected for high swim-induced aerobic metabolism ("aerobic" A lines). In generation 21, voles from the 4 A lines achieved a 57% higher "voluntary maximum" swim-induced aerobic metabolism (VO_2swim) than voles from 4 unselected, "control" C lines. In C lines, VO_2swim was 9% lower than the maximum forced-exercise aerobic metabolism (VO_2run; $P = 0.007$), while in A lines it was even higher than VO_2run, although not significantly (4%, $P = 0.15$). Thus, we hypothesized that selection changed both the aerobic capacity and the neuronal mechanisms behind motivation to undertake activity. We investigated the influence of reuptake inhibitors of dopamine (DARI), serotonin (SSRI), and norepinephrine (NERI) on VO_2swim. The drugs decreased VO_2swim both in C and A lines (% decrease compared with saline: DARI 8%, $P < 0.001$; SSRI 6%, $P < 0.001$; NERI 8%, $P < 0.001$), but the proportional response differed between selection directions only for NERI (stronger effect in C lines: $P = 0.008$) and the difference was marginally non-significant for SSRI ($P = 0.07$) and DARI ($P = 0.06$). Thus, the results suggest that all the 3 monoamines are involved in signaling pathways controlling the motivation to be active and that norepinephrine could have played a role in the evolution of increased aerobic exercise performance in our animal model.

Key words: experimental evolution, monoamines, motivation, physical activity, selective breeding, voluntary exercise

The actual level of physical activity of an organism depends on both its physical and physiological abilities (e.g., muscle strength) and behavioral characteristics (e.g., motivation to perform exercise; Good et al. 2015; Garland et al. 2011). Many studies highlight importance of the latter in the physical activity of humans and other animals, but the neurobiological basis of increased voluntary physical activity is not fully recognized (see review: Garland et al. 2011; de Geus et al. 2014; Rosenfeld 2017). Understanding the mechanisms is crucial for preventing alarmingly growing prevalence of several civilization diseases resulting from low levels of physical activity of humans (Bauman et al. 2012). Neuronal factors underlying the motivation to involve into physical activity or to perform at the upper level of physical abilities are also of interest in evolutionary science (Lightfoot 2013). It has been proposed that evolution of performance traits is driven by natural selection operating primarily on behavior, but the range of behaviors is limited by animals' performance, and therefore selection impels subsequent morpho-physiological changes underlying the performance (Garland and

Carter 1994). As survival and reproductive success depend on behaviors that usually require increased physical activity (e.g., mating, foraging, avoiding predators; Ekkekakis et al. 2005), neurobiological rewards (e.g., "the runners high" pleasurable feeling of euphoria after running) could play a role in the evolution of increased performance (Ekkekakis et al. 2005).

In this study, we tested the hypothesis that evolution of an increased aerobic exercise performance can be facilitated by evolution of motivation to undertake physical activity. We use the term "motivation" for all behavioral aspects regulating the level of physical activity, as opposed to physical "ability" (Garland et al. 2011; Good et al. 2015; Brellenthin et al. 2017). We used a unique animal model: bank voles from a selection experiment (Sadowska et al. 2008, 2015; Jaromin et al. 2016). After 19 generations of selection for high swim-induced aerobic metabolism (VO_2swim) voles from the A-selected lines ("Aerobic") achieved a 61% higher mass-adjusted metabolism than those from unselected C lines ("Control"; Jaromin et al. 2016; Supplementary Figure S1.1). This difference in VO_2swim remains significant through the whole life of the voles (Rudolf et al. 2017). Voles from the A lines have also a higher basal metabolic rate (Sadowska et al. 2015) and are more active in open-field test than voles from the C lines (Maiti et al. 2018, accepted for publication). A whole transcriptome analysis indicated several putative candidate genes with an altered expression level or single-nucleotide polymorphisms (SNP) frequency, and revealed that the selection differences were mostly due to the changes in expression rather than changes in SNP frequencies (Konczal et al. 2016, 2015). Noteworthy, the VO_2swim may not be *the maximum* metabolism *per se* (the aerobic capacity), because the voles can float on the water surface, and therefore are not forced to use their maximal abilities. Thus, VO_2swim can be treated as a measure of the maximum voluntary intensity of the exercise. Not surprisingly, in generation 5, VO_2swim in both A and C lines was about 23% lower than the maximum forced-running rate of aerobic metabolism (VO_2run; Supplementary Table S1.1). Presumably, the external motivation to undertake vigorous activity applied during forced running trial (electric shocks) is higher than that during swimming (in warm water). In generation 19, however, C-line voles still achieved 19% lower VO_2swim than VO_2run, while A-line voles achieved similar VO_2 during swimming and running (Jaromin et al. 2016). Thus, it seems that both the physical abilities and behavior (motivation to exercise) evolved in our model system. We hypothesize that in the A lines signaling of neurotransmitters involved in high motivation increased, or signaling associated with loss of motivation to exercise decreased.

Physical activity is regulated by a complex network, which includes, among others, neuronal pathways associated with monoamine neurotransmitters. For instance, dopamine is implicated in motivation, rewarding, and motor movement (Vallone et al. 2000; Knab and Lightfoot 2010). Serotonin is involved in regulation of fatigue and locomotion (Davis and Bailey 1997). Norepinephrine regulates the level of arousal, reward mechanisms, and consciousness (Roelands and Meeusen 2010). According to the central fatigue hypothesis, these monoamines, and especially changes in their concentration ratios, influence the drive to be active and determine the capacity to perform exercise (Roelands and Meeusen 2010).

To investigate differences between the A and C lines in the monoaminergic signaling pathways we used a pharmacological approach, based on the assumption that a change in neurophysiological mechanism underlying a behavioral trait alters a behavioral or physiological response to pharmacological agents. This approach (i.e., manipulation of a neurotransmission by systemic drug administration) is widely applied to investigate the involvement of monoamines in exercise performance of both animals and humans (review in Roelands and Meeusen 2010). It is especially useful when a large number of animals has to be tested (which may practically preclude applying more sophisticated neurophysiological methods), as it is in the case of selective breeding experiments. The approach has been already successfully used within the framework of other selection experiments (e.g., Rhodes et al. 2001; Rhodes and Garland 2003; Keeney et al. 2008, 2012). For instance, administration of a dopamine reuptake inhibitor (RI) (a drug that effectively increases the dopaminergic signaling) resulted in decreased activity of mice selected for high wheel-running behavior, but had little effect on unselected, control mice (Rhodes et al. 2001). This differential response suggested differences in dopamine signaling between high wheel-runners and control mice. Indeed, the level of brain dopamine in high wheel-runners was decreased (Waters et al. 2013). Similarly, hyperactivity of patients with dopamine signaling impairment (Attention Deficit Hyperactivity Disease, ADHD) is decreased after stimulants administration, while the drugs usually increase the activity of a healthy person (Jafarinia et al. 2012). Our previous work showed no effect of bupropion (a non-selective dopamine and norepinephrine RI) on aerobic performance of bank voles (Jaromin et al. 2016). In this study, instead of nonselective drugs (such as amphetamine or cocaine), we used selective agents to investigate potential changes in particular neurotransmission: selective RIs of dopamine (DARI, vanoxerine), serotonin (SSRI, fluoxetine), and norepinephrine (NERI, reboxetine). As these drugs exert mainly central monoaminergic effects, a distinct reaction to drugs administration in the A and C lines will indicate that a particular neurotransmitter signaling pathway has been changed due to selection (Rhodes et al. 2001; Rhodes and Garland 2003; Keeney et al. 2008, 2012).

Material and Methods

Animals

We used bank voles *Myodes glareolus* from the artificial selection experiment, in which 4 replicate lines of voles are selected for the 1-min maximum rate of oxygen consumption achieved during 18-min swimming trial at 38°C (VO_2swim; "A"—aerobic lines) and 4 replicate lines of unselected, randomly bred voles are maintained as control ("C"). The rationale of the entire experimental evolution project and the choice of bank voles as the model organism, details of the selection procedure, and animal maintenance are described in Sadowska et al. (2008, 2015). In generation 21, we measured VO_2swim in 1032 A-line voles and 259 C-line voles at the age of 75–85 days as a part of the regular selection protocol (selection trial). The A-line voles with the highest VO_2swim were used for reproduction and could not be included in the pharmacological experiment. Thus, to avoid the bias, we excluded also A-line voles that achieved the lowest VO_2swim, and chose C-line voles from the middle of the distribution, too. Voles that could not swim or were diving were excluded. In that way, we chose 6 females and 6 males from each of the A and C lines (a total of 96, each representing a different family) for each of the 2 experiments (with DARI and with SSRI/NERI). The voles were assigned to 3 blocks in each experiment (2 voles from each replicate line and sex in 1 block, tested on the same day).

Pharmacological experiments

At the age of 100–165 days the voles underwent a series of 3 post-injection VO_2swim measurements. We applied the specific RIs of

dopamine (vanoxerine), noradrenaline (reboxetine), and serotonin (fluoxetine) that, according to literature, influence physical activity in many different rodent species, including bank voles (fluoxetine only: Meers and Ödberg 2005; Korff et al. 2008). These RIs were also successfully used in similar studies concerning the comparison between lines of rodents selected for locomotor performance traits (Wong et al. 2000; Rhodes et al. 2001; Cryan et al. 2005a, 2005b; Meers and Ödberg 2005; Korff et al. 2008; Esumi et al. 2013). The exact doses were chosen based on results of pilot trials.

Because of pioneering nature of our animal model, we preceded the main experiment with pilot trials, in which we assessed the effect of the drugs administration on spontaneous activity of voles in their home cages. The aim of the pilot trials was to exclude the possibility that the dosages of the drugs applied in other studies were too high for bank voles (e.g., causing immediate sedation, which would preclude swimming trials) or too low and not resulting in any detectable change of behavior. We used 4 females and 4 males from each of the A and C lines in a repeated measures design. During subsequent trials, each individual was injected intraperitoneally with either vanoxerine (DARI; 10 mg/kg or 20 mg/kg), reboxetine (NERI; 10 mg/kg or 20 mg/kg), fluoxetine (SSRI; 10 mg/kg or 20 mg/kg), or saline (a control). Additionally, each individual was injected with a less specific RI of dopamine and noradrenaline (bupropion; 20 mg/kg, 30 mg/kg) as a preparation for another experiment (Jaromin et al. 2016). The injections were counterbalanced and administered in random order. We maintained 3-day breaks between the trials to avoid a carry-over effect. A complete description of the measurement protocol, statistical analyses, and results of the pilot trials is presented in Supplementary Materials. Shortly, DARI in the low dose increased and in high dose decreased the activity, although the effect of the low dose was not significant (low dose: $P = 0.87$, high dose: $P = 0.007$; Supplementary Figure S2.1 and Table S2.3). We observed a significant decrease of activity after injection of both doses of NERI ($P < 0.001$) and the high dose of SSRI (low dose: $P = 0.94$, high dose: $P < 0.001$).

In the proper study, to minimize the number of trials repeated on the same individual (and hence an effect of training), we performed 2 experiments on separate groups of animals: 1 with DARI treatment and the other with NERI and SSRI treatment. In each of the 2 experiments 3 swimming trials on each individual were performed (i.e., the repeated measures design was applied). As some dopamine RIs can give a differential effect depending on the dose administered, for example, they increase activity in low dose and decrease activity in high dose (e.g., Niculescu et al. 2005), and we also observed such a pattern in the pilot trials (although the effect of the low dose was not significant), we used 2 doses of DARI in the first experiment. However, to strengthen the possibly differential response to DARI, we decreased the low dose (to 5 mg/kg from 10 mg/kg used in the pilot) and increased the high dose (to 25 mg/kg from 20 mg/kg). In the second experiment, we chose the high dose of SSRI, as this dose significantly decreased activity in the pilot trials. However, as both doses of NERI decreased the voles activity in the pilot trials to a similar level (Supplementary Figure S2.1), we chose the lower dose to avoid side-effects that are more likely to occur in the case of too high a dose, that is, an overdose (e.g., Whiskey and Taylor 2013). Thus, in the experiment with DARI, animals received injections of either saline, or DARI in low (5 mg/kg) or high dose (25 mg/kg). In the experiment with SSRI/NERI, animals received injections of either saline, or SSRI 20 mg/kg or NERI 10 mg/kg. Each individual received 1 injection in 1 of the 3 measurement days. The injections were administered in a random order, but we balanced the number of each kind of injection administered to voles from a particular line in each trial. To avoid carry-over effects, we maintained a 1 week break between the repeated trials. All injections were administered intraperitoneally, in a volume of 10 mL/kg, 30 min before the VO_2swim measurement. Vanoxerine hydrochloride, fluoxetine hydrochloride, and reboxetine mesylate (Cayman Europe, Tallinn, Estonia) were diluted in 0.9% saline (vanoxerine and fluoxetine required sonification for 45 min–2 h in 40°C) and filtered with syringe filters with micropores 0.2 µm (Rotilabo, CarlRoth). The solutions were freshly prepared every day, ca. 2 h before the trials.

Measurement techniques

The rate of oxygen consumption (VO_2) was measured with open-flow respirometric systems. We used 2 similar systems for the measurement of swim-induced VO_2 (VO_2swim). The respirometric chambers (a 15 cm diameter 3-L jar) were partly filled with water (38°C). The air flow (2 L/min) was controlled with mass-flow controllers (GFC17 or GFC171S, Aalborg, Orangeburg, NY, USA). A sample of excurrent air was dried with ND2 gas sample drier (Sable Systems, Las Vegas, NV, USA) and with chemical absorber (magnesium perchlorate), and directed to FC10 or FC-10a oxygen and CA-2A CO_2 analyzers (Sable system, Las Vegas, NV, USA), which recorded the gas concentrations at 1-s intervals. The rates of oxygen consumption were calculated with appropriate respirometric equations and corrected for "effective volume" to achieve "instantaneous" rates (Lighton 2008).

Behavior of the voles during the swimming trials was recorded by waterproof cameras placed at the bottom of the chambers. Recordings were analyzed in JWatcher version 1.0 by an observer unaware of the treatment and selection direction for each animal. The voles were scored as inactive ("immobility time": floating motionless or doing only small movements to keep nose above the water surface) or active as either "swimming" (swimming around the chamber in horizontal position, with forepaws usually not moving), "climbing" (swimming in upright position, with forepaws moving partly above the water surface), "diving" (apparently determined swimming under water), or "drowning" (apparently uncontrolled submergence). As the last 2 categories were rare, and because "climbing" behavior is especially interesting in the context of such tests (see the "Discussion" section), statistical data analyses were performed only for "proportion of activity" (the total activity time divided by total trial time) and for "proportion of climbing" (climbing time divided by the total activity time).

About 2 weeks after the pharmacological trials, we measured the 1-min maximum forced-running rate of oxygen consumption (VO_2run) in a respirometric treadmill for rodents (BTU-100-10-M, Bio-Sys-Tech, Bialystok, Poland). The animals were forced to run with mild electric shocks (0.5 mA) generated by bars located behind the moving belt. The fur of the animal's abdomen and hind legs was moistened with water with a drop of dog shampoo, to increase electric conductivity. Without that procedure the animals ignored the electric shocks. The treadmill started to move at 5 m/min 1 min after starting the trial and the speed was increased by 5 m/min every minute. The test lasted untill exhaustion, that is, untill the animal was unable to keep pace with the moving belt (typically 5–15 min). To decrease velocity at which the voles achieved the maximum effort (typically 25–75 m/min), and hence make the procedure safer to the animals, the treadmill was inclined by 10°. We also placed 2 ping-pong balls at the end of the moving belt, which helped the animals learn faster to avoid the bars. The measurements were

preceded by 2 habituation trials to familiarize the animals with the treadmill. The habituation trials were performed in the same conditions as the proper measurements. The method of oxygen and CO_2 recording and of calculating VO_2run was the same as for VO_2swim.

All procedures associated with the breeding, selection, and experimental procedures were approved by the Local Ethical Committee in Krakow, Poland (No. 68/2012 and No. 61/2014).

Statistical analyses

To analyze the data we used nested analysis of covariance (ANCOVA) mixed models implemented in SAS 9.3 (SAS Institute Inc., Cary, NC, USA) Mixed procedure (with REML estimation method and variance components constrained to non-negative; SAS Institute Inc., 2011). As the whole analyses included several complex models, we provide—in addition to the verbal description presented below—a Supplementary Material with a commented SAS code used for the analyses.

The models used for analyzing the selection-trial body mass and VO_2swim included the following main fixed factors: line Type (selected, control), Sex, and line Type × Sex interaction. The models included also random effects (replicate Line nested in line Type, Sex × Line interaction), fixed covariates (Age, litter Size), and cofactors (Number of the litter of a female). In addition, the model for VO_2swim included Body mass as a covariate and Respirometric system number as a cofactor. In the analyses performed for all voles from generation 21 we included also a random effect of Family nested in Line (the effect was not included in models for the pharmacological trials described in the next paragraph, because each individual represented a different family).

To analyze data from the 3 subsequent pharmacological trials we used similar ANCOVAs, but in repeated-measures design. The analyses were performed for the following dependent variables: 1-min maximum VO_2swim, time of achieving the 1-min maximum VO_2swim, mean VO_2swim (mean VO_2swim calculated for the whole trial), proportion of activity, proportion of "climbing," and proportional response (the ratio of VO_2swim after drug to that after saline). These models included the same between-subjects effects as described above (plus measurement Block as an additional random effect), and the following within-subject (within-individual) effects: fixed factors of a Drug (DARI experiment: DARI low dose vs. DARI high dose vs. saline; SSRI/NERI experiment: SSRI vs. NERI vs. saline), line Type × Drug interaction and trial Number (a repeated measure factor), and random effect of Drug×Line interaction. The proportion of activity was arcsine-transformed (arcsine of the square root of a value). Based on Akaike Information Criteria (AIC), we chose compound symmetry as the best-fitted covariance structure.

A repeated measures ANCOVA was also used to compare VO_2swim (after saline injection only) with VO_2run. The model included the same between-subjects effects as described above and the following within-subject effects: fixed factors of Exercise type (swim vs. run; a repeated measure factor) and Exercise type × line Type interaction, and random effect of Line × Exercise type. We performed this analysis on pooled data from both pharmacological experiments, so we included Experiment type as an additional cofactor and random effect of Family nested in Line.

All initial models included interactions between Sex and other main factors. However, these effects were not of the main interest and were excluded from the final models when not significant ($P > 0.05$). To test for the homogeneity of slopes we included interactions between Body mass and main factors, which were excluded from the final models when not significant. However, the slopes of the relationships between oxygen consumption rate (VO_2) and Body mass were always steeper in A than in C lines, and that difference was significant in some analyses. To investigate whether the levels of VO_2 differ between the line Types in the range of observed body mass, we tested this effect also at specific values of the mass (option "at" in LSMEANS statement in the SAS Mixed procedure; SAS Institute Inc., 2011). For a more transparent presentation of the results, the adjusted least-squares means (LSM) presented on figures were calculated at mean values of covariates (body mass and age: 24.0 g and 84 days in selection trial; 24.6 g and 136 days in further trials; litter size: 6).

In all models, we used the Satterthwaite's approximation to calculate the effective denominator degrees of freedom (dfs) of the *F*-statistics (SAS Institute Inc., 2011). Shortly, in the model with nested random effects, the appropriate denominator dfs are computed as combination of the dfs of appropriate random effects (i.e., the nested factor effects and residual term) weighted by variance contributed by the effects. When the variance of the nested factor (in our case replicate Lines nested in line Type) approaches zero, the appropriate denominator df approach dfs for the residual error term (which reflects the fact that in the absence of the among-group variance, individual observations can be effectively treated as independent). Thus, when the Satterthwaite method is used, the denominator dfs can take any value from the range between the df of the nested random factor and the df of residual term.

Unless otherwise stated, we used Tukey–Kramer for multiple pairwise comparisons between groups or Dunnett comparisons between saline-drug groups. In all the models variance was constrained to positive values. We tested the significance of random factors with likelihood ratio test. For that purpose we fitted models such as described above, but with the variance constraint relaxed ("noboud" option in the Proc Mixed statement in SAS; SAS Institute Inc., 2011).

In the "Results" section, we report main effects of line Type and Drug. For descriptive statistics and detailed information about all others effects see Supplementary Tables S1.2–S1.7.

Results

Effects of selection

Body mass in the selection trial ranged from 13.4 g to 38 g (mean: 24 g; Figure 1A and Supplementary Table S1.2). Body mass did not differ significantly between A and C lines ($F_{1,6} = 2.91$, $P = 0.14$). Males were heavier than females and line Type × Sex interactions were not significant (Supplementary Table S1.4).

Both the swim-induced (VO_2swim) and run-induced (VO_2run) oxygen consumption increased with body mass (Figure 1 and Supplementary Table S1.3). For VO_2swim, the slope of the relationship was higher in A than in C lines, and the mass-adjusted VO_2swim was significantly higher in A than in C lines at the minimum body mass ($P < 0.0001$), which implies that it was also true in the entire range of body mass. In A lines the levels of VO_2swim and VO_2run were similar ($t_7 = 2.47$, $P = 0.15$), while in C lines VO_2swim was lower than VO_2run ($t_7 = 4.91$, $P = 0.007$; overall ANCOVA: Exercise type: $F_{1,7} = 2.84$, $P = 0.14$, line Type × Exercise type: $F_{1,7} = 27.1$, $P = 0.001$; Figures 1B and 2). Voles from the selected lines had also higher values of mean VO_2swim, proportion of activity time, and proportion of "climbing" behavior during the swimming trials (Figures 3 and 4, and Table 1; details of the results are presented below).

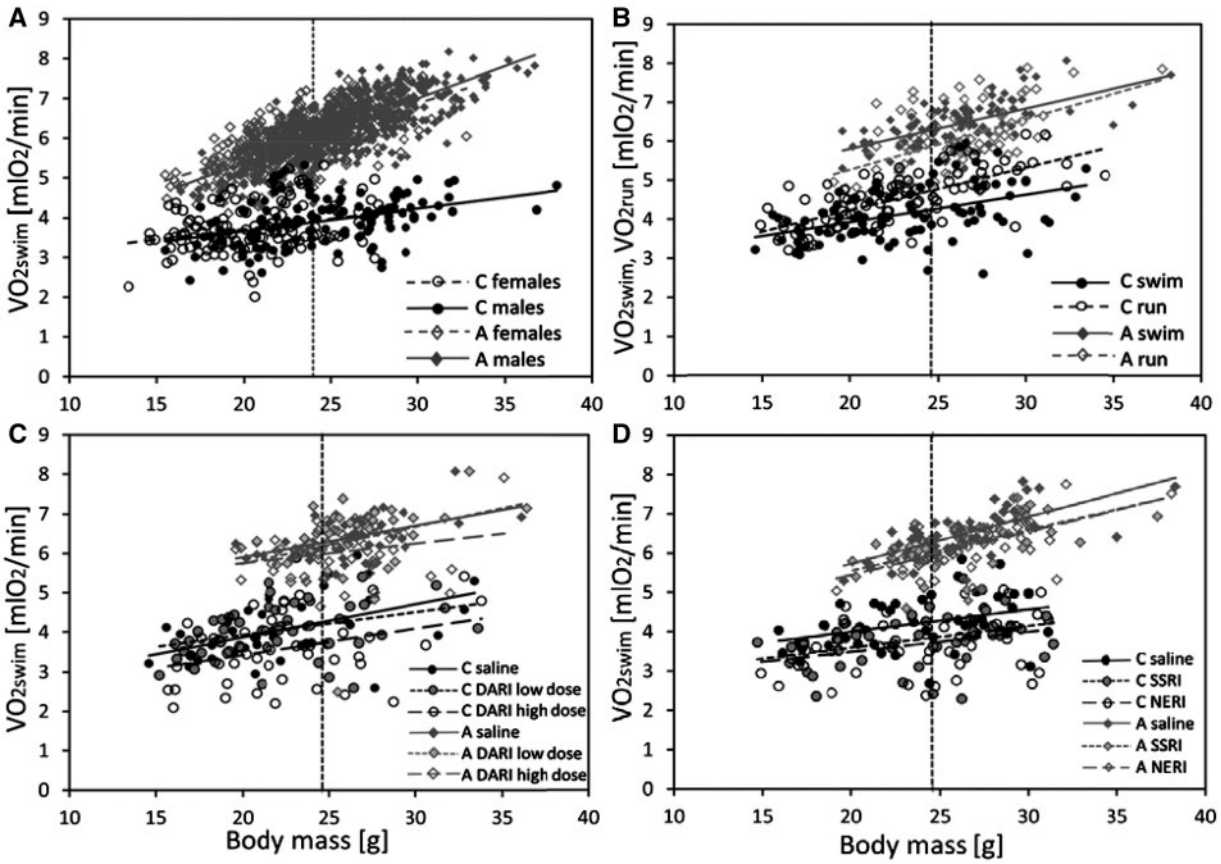

Figure 1. The relationship between swim-induced (VO_2swim) or run-induced (VO_2run) 1-min maximum rate of oxygen consumption and body mass in bank voles, and the effects of selection, sex, and pharmacological manipulation on the level of VO_2swim. (**A**) VO_2swim in all individuals from generation 21 (C, Control lines; A, Aerobic lines) tested as a part of the selection experiment (selection-trial results). (**B**) The effect of Exercise type (VO_2swim vs. VO_2run) in voles from C ($N=96$) and A ($N=96$) lines (voles used in DARI and NERI/SSRI experiments combined). (**C**) The effect of DARI (vanoxerine) in low and high dose on VO_2swim: repeated trials in a subsample of voles from C ($N=48$) and A ($N=48$) lines. (**D**) The effect of SSRI (fluoxetine) and NERI (reboxetine) on VO_2swim: repeated trials in a subsample of voles from C ($N=48$) and A ($N=48$) lines. Dotted lines indicate the mean body mass for which the adjusted least-squares means were calculated (shown in Table 1 and Figures 2–4).

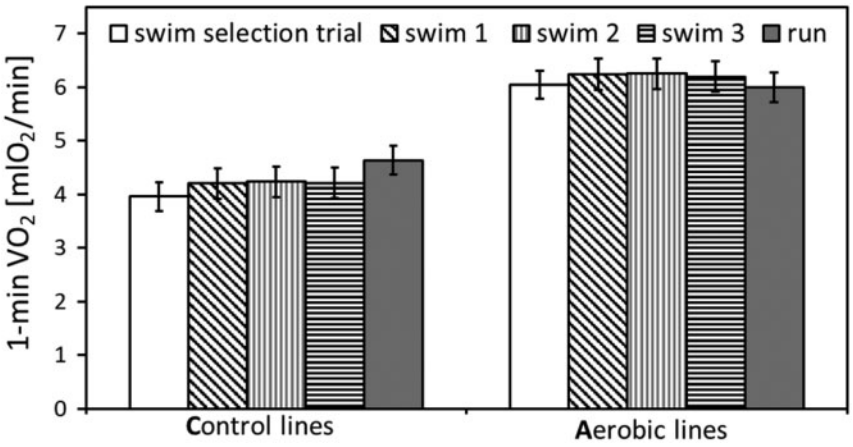

Figure 2. The swim-induced rate of oxygen consumption achieved during the selection trial and pharmacological trials (swim trials 1–3 after saline injections only), and the maximum forced-exercise (running) rate of oxygen consumption in bank voles from selected, aerobic A lines ($N=96$) and unselected, control C lines ($N=96$). The adjusted least squares means for the mean body mass (24 g) with 95% confidence limits (LSM [95% CL]; note: an overlap of the confidence limits does not indicate a lack of difference between repeated measurements at different conditions, because the confidence limits are based on among-individual variation at particular conditions, whereas the inferences concerning differences between conditions are based on within-individual comparisons. See Table 1 for results of the proper significance tests).

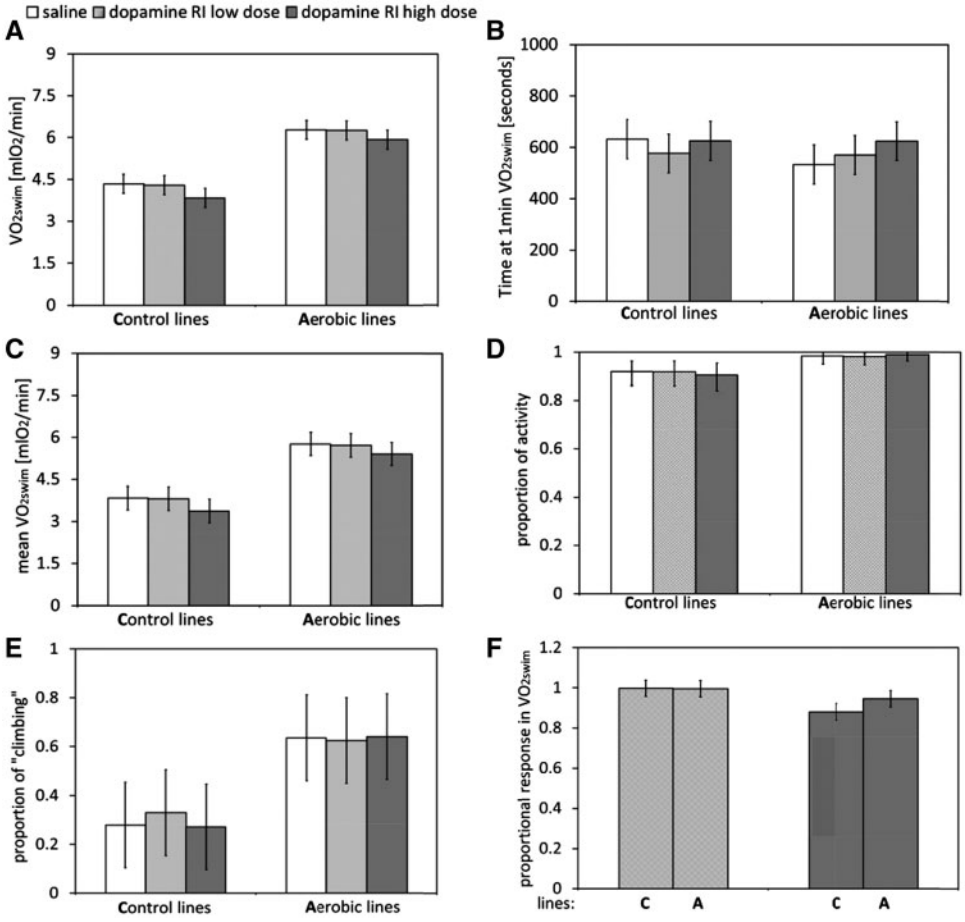

Figure 3. Summary of the main results from experiment with DARI (vanoxerine) in low and high dose: repeated trials in a subsample of voles from selected A ($N = 48$) and unselected C lines ($N = 48$). (**A**) The swim-induced 1-min maximum rate of oxygen consumption (VO_2swim). (**B**) The time when voles achieved the VO_2swim. (**C**) Whole-trial mean VO_2swim (typically about 15 min). (**D**) Duration of activity divided by whole trial duration (proportion of activity); back-transformed least squared means. (**E**) Duration of climbing divided by duration of activity (proportion of "climbing"). (**F**) The ratio of VO_2swim achieved after drug to the one after saline (proportional response). Bars represent adjusted least squares mean with 95% confidence limits (LSM [95% CL]; note: as explained in Figure 2 legend, an overlap of the confidence limits does not indicate that a difference is not significant. See Figure 5 and Table 1 for results of the proper significance tests).

DARI experiment

The 1-min maximum VO_2swim increased through the 3 subsequent trials (trial 1 vs. trial 2: $t_{186} = 2.93$, $P = 0.01$, trial 2 vs. trial 3: $t_{184} = 1.60$, $P = 0.25$, trial 1 vs. trial 3: $t_{187} = 4.46$, $P < 0.001$; Supplementary Table S1.5). The VO_2swim was higher in A than C lines ($P < 0.0001$; Figures 1C and 3A, and Table 1). Only the high DARI dose decreased VO_2swim (compared with saline: Low Dose: $t_{184} = 0.54$, $P = 0.81$, High Dose: $t_{185} = 7.09$, $P < 0.0001$, Figures 3A and 5A), but the line Type × Dose interaction was not significant ($P = 0.35$). As DARI in the Low Dose had practically no effect, we performed a separate analysis only for the high dose, but the line Type × Dose interaction remained not significant ($F_{1,91} = 1.47$, $P = 0.23$). The time of achieving VO_2swim did not depend on line Type, Dose, or line Type × Dose interaction ($P > 0.24$; Figures 3B and 5B). As in the analysis of 1-min maximum VO_2swim, whole-trial mean VO_2swim (typically about 16 min) was higher in A than in C lines and decreased after the high dose, but line Type × Dose interaction was not significant (Figures 3C and 5C, and Table 1).

The proportion of activity time decreased in the third trial when compared with the first and second trail (trial 1 vs. trial 2: $t_{134} = 0.09$, $P = 0.99$, trial 2 vs. trial 3: $t_{133} = 3.55$, $P = 0.001$, trial 1 vs. trial 3: $t_{126} = 3.67$, $P = 0.001$; Supplementary Table S1.5). Voles from A lines were more active than those from C lines ($P = 0.02$; Figure 3D and Table 1) and had a higher proportion of "climbing" ($P = 0.008$; Figure 3E). The drug did not influence significantly either the overall activity or the proportion of "climbing" ($P > 0.18$; Figure 5D,E).

Note that in the additive models (results reported above) the line Type × Dose interactions conveys the information on the effect of selection on monoamines pathway. However, in the analysis of the proportional response to the drug (the ratio of VO_2swim after drug to that after saline) the effect of selection on monoamines pathway is expressed directly in the line Type effect. The overall line Type effect was not significant ($P = 0.19$; Figure 3F and Supplementary Table S1.6), but the proportional response to the High Dose was much stronger than to the Low Dose ($P = 0.0009$; Figure 5F), and the line Type × Dose interaction was nearly significant ($P = 0.06$). An analysis performed separately for the 2 doses showed that the proportional response to High Dose tended to be stronger in C than in A lines (C vs. A comparisons with Sidak correction: Low Dose: $F_{1,18} = 0.01$, $P = 1$, High Dose: $F_{1,18} = 5.43$, $P = 0.06$; Figure 5F).

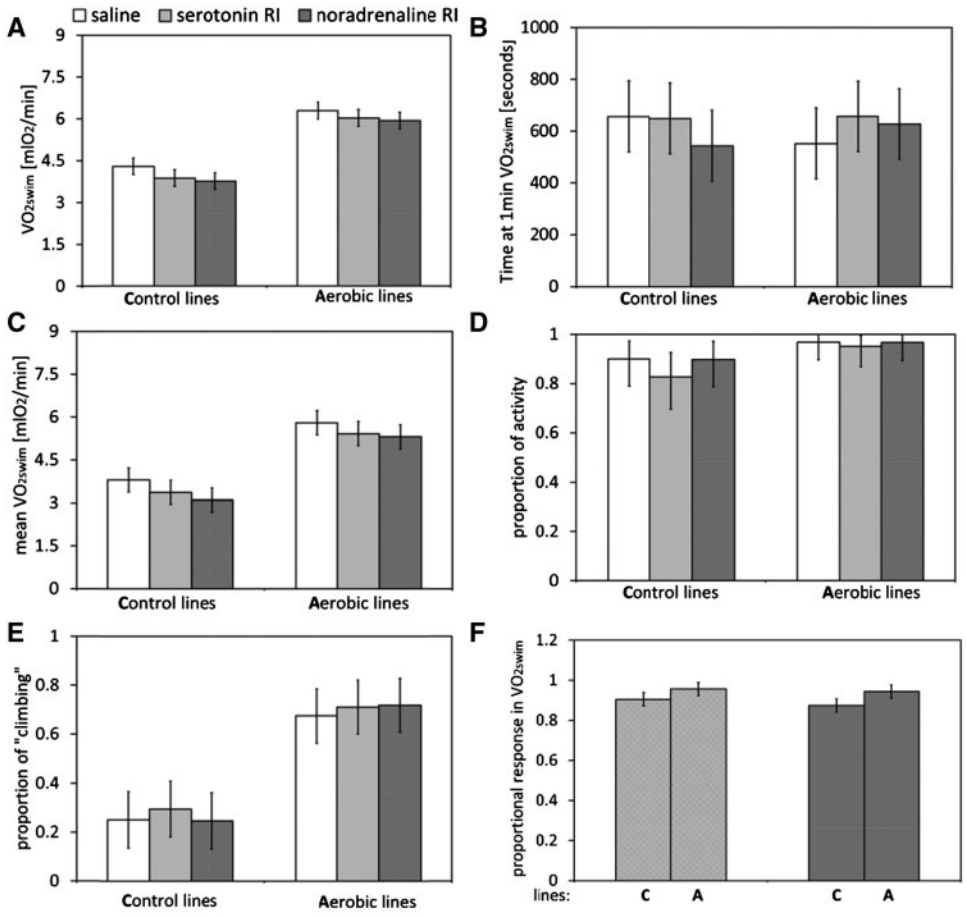

Figure 4. Summary of the main results from experiment with SSRI (fluoxetine) and NERI (reboxetine): repeated trials in a subsample of voles from selected A ($N = 48$) and unselected C lines ($N = 48$). (**A**) The swim-induced 1-min maximum rate of oxygen consumption (VO_2swim). (**B**) The time when voles achieved the VO_2swim. (**C**) Whole-trial mean VO_2swim (typically about 15 min). (**D**) Duration of activity divided by whole trial duration (proportion of activity); back-transformed least squared means. (**E**) Duration of climbing divided by duration of activity (proportion of "climbing"). (**F**) The ratio of VO_2swim achieved after drug to the one after saline (proportional response). Bars represent adjusted least squares mean with 95% confidence limits (LSM [95% CL]; note: as explained in Figure 2 legend, an overlap of the confidence limits does not indicate that a difference is not significant. See Figure 5 and Table 1 for results of the proper significance tests).

SSRI/NERI experiment

The 1-min maximum VO_2swim increased through the 3 subsequent trials (trial 1 vs. trial 2: $t_{176} = 3.97$, $P = 0.003$, trial 2 vs. trial 3: $t_{174} = 0.75$, $P = 0.73$, trial 1 vs. trial 3: $t_{173} = 3.28$, $P = 0.004$; Supplementary Table S1.7). The VO2swim was higher in A than C lines ($P < 0.0001$; Figures 1D and 4A, and Table 1). Both drugs decreased VO_2swim (compared with saline: SSRI: $t_{12} = 5.63$, $P = 0.0001$, NERI: $t_{11} = 7.36$, $P < 0.0001$; Figure 5A), but the line Type × Drug interaction was not significant ($P = 0.31$). The time of achieving VO_2swim was not significantly affected by the line Type ($P = 0.95$; Figure 4B), but there was a significant line Type × Drug interaction ($P = 0.026$). Specifically, in C lines the time of achieving VO_2swim tended to be shorter after NERI (within line comparisons of drug vs. saline, with Sidak correction; $F_{1,185} = 5.40$, $P = 0.08$) and was not affected by SSRI ($F_{1,189} = 1.96$, $P = 0.51$), whereas A lines tended to achieve VO_2swim later after injections of NERI ($F_{1,190} = 2.34$, $P = 0.42$) and SSRI ($F_{1,190} = 4.45$, $P = 0.14$). As in the analysis of 1-min maximum VO_2swim, whole-trial mean VO_2swim was higher in A than in C lines and decreased after the drugs injections, but line Type × Drug interaction was not significant (Figures 4C and 5C, and Table 1).

The proportion of activity time decreased in the third trial when compared with the first and second trail (trial 1 vs. trial 2: $t_{186} = 0.92$, $P = 0.63$, trial 2 vs. trial 3: $t_{187} = 3.26$, $P = 0.004$, trial 1 vs. trial 3: $t_{187} = 2.37$, $P = 0.05$; Supplementary Table S1.7), and tended to be higher in A than in C lines ($P = 0.09$; Figure 4D and Table 1). The proportion was lower after SSRI than saline injection ($t_{188} = 4.05$, $P < 0.0001$; Figure 5D), but it was not affected by NERI ($t_{188} = 0.23$, $P = 0.96$) or line Type × Drug interaction ($P = 0.13$). The proportion of "climbing" increased through subsequent trials (trial 1 vs. trial 2: $t_{175} = 2.61$, $P = 0.03$, trial 2 vs. trial3: $t_{175} = 1.48$, $P = 0.30$, trial 1 vs. trial 3: $t_{175} = 4.09$, $P = 0.0002$; Supplementary Table S1.7) and was higher in A than in C lines ($P = 0.0002$; Figure 4E and Table 1), but was not affected by the drugs (Drug: $P = 0.32$, line Type × Drug: $P = 0.51$; Figure 5E).

The proportional response to drugs (the ratio of VO_2swim after drug to that after saline) was stronger in C than in A lines (line Type: $P = 0.004$; Figure 5F), but the difference for SSRI was marginally non-significant (C vs. A comparisons with Sidak correction: NERI: $F_{1,129} = 9.07$, $P = 0.008$, SSRI: $F_{1,132} = 4.89$, $P = 0.07$; Figure 4F and Supplementary Table S1.6). The Drug effect was

Table 1. Main results of mixed ANCOVA models (effects of selection, pharmacological treatment, and their interaction) for the data obtained in DARI and SSRI/NERI experiments

Experiment	Line Type	Dose/Drug	Line Type × Drug
Trait	*t* (df), *P*-value	*F* (Ndf, Ddf), *P*-value	*F* (Ndf, Ddf), *P*-value
DARI experiment			
VO_2swim	10.0 (7), <0.0001	31.6 (2, 185), <0.0001	1.03 (2, 185), 0.36
Time of achieving VO_2swim	0.95 (12), 0.36	1.60 (2, 14), 0.24	1.59 (2, 14), 0.24
Mean VO_2swim	7.93 (7), 0.0001	22.8 (2, 11), 0.0001	0.6 (2, 11), 0.58
Proportion of activity	3.22 (6), 0.02	0.1 (2, 91), 0.89	1.72 (2, 91), 0.18
Proportion of "climbing"	3.71 (7), 0.008	0.3 (2, 176), 0.70	1.00 (2, 176), 0.38
Proportional response	1.35 (29), 0.19	30.6 (1, 7), 0.0009	4.87 (1, 7), 0.06
SSRI/NERI experiment			
VO_2swim	12.2 (7), <0.0001	29.6 (2, 11), <0.0001	1.31 (2, 11), 0.31
Time of achieving VO_2swim	0.06 (7), 0.95	2.01 (2, 188), 0.14	3.73 (2, 188), 0.03
Mean VO_2swim	8.48 (6), 0.0001	48.6 (2, 11), <0.0001	1.50 (2, 11), 0.26
Proportion of activity	1.95 (6), 0.10	10.5 (2, 188), <0.0001	2.03 (2, 188), 0.13
Proportion of "climbing"	6.52 (8), 0.0002	1.26 (2, 11), 0.32	0.7 (2, 11), 0.51
Proportional response	2.99 (81), 0.004	3.29 (1, 85), 0.07	0.6 (1, 85), 0.43

Notes: VO_2swim, 1-min maximum swim-induced rate of oxygen consumption; mean VO_2swim, whole-trial mean VO_2swim; proportional response, the ratio of VO_2swim achieved after drug to the one after saline; note that in the proportional response analysis the line Type × Drug interaction has only one degree of freedom and has different meaning than in the other analyses (see the sections "Materials and Methods" and "Discussion" for details); *F*- or *t*-statistics; df, degrees of freedom; ndf, numerator df; ddf, denominator df; see Supplementary Tables S1.5 and S1.7 for results concerning additional factors in the ANCOVA models.

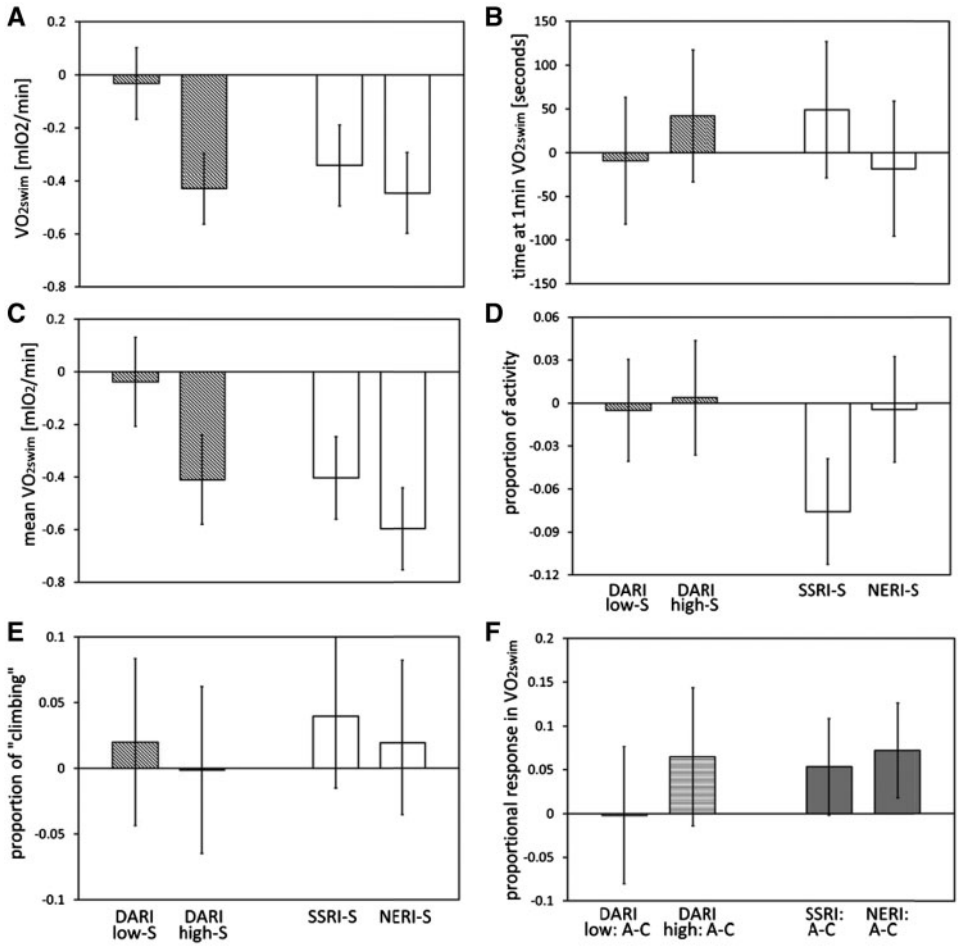

Figure 5. The effect of DARI (vanoxerine in low and high dose), SSRI (fluoxetine), and NERI (reboxetine) on the measured traits. (**A–E**) Mean within-individual differences between the values obtained after drug treatment and saline injection (A, the swim-induced 1-min maximum rate of oxygen consumption [VO_2swim]; B, the time when voles achieved the VO_2swim; C, whole-trial mean VO_2swim; D, duration of activity divided by whole trial duration; E, Duration of "climbing" divided by duration of activity). (**F**) Difference between the A and C lines in the ratio of VO_2swim achieved after drug to that after saline (proportional response). Bars represent the least squares estimates of the mean differences with 95% confidence limits.

marginally non-significant ($P = 0.07$) and line Type × Drug interaction was not significant ($P = 0.43$).

Discussion

As expected, in generation 21 of the selection experiment bank voles from the A-selected lines achieved more than 57% higher swim-induced aerobic metabolism (VO_2swim) than those from the unselected C-control lines (Figure 1A). As in our previous work, performed on generation 19 (Jaromin et al. 2016), VO_2swim was 9% lower than the maximum forced-running aerobic metabolism (VO_2run) in C lines, but in the A lines the levels of VO_2swim and VO_2run were practically the same (Figures 1B and 2). Thus, A-line voles evolved both an increased aerobic capacity and an increased motivation to be physically active. Observations of behavior showed that during the swimming trials A-line voles spent more time on active behavior, especially more time on "climbing," than C-line voles (Figures 3D,E and 4D,E). Such a profile is characteristic for animal models of reduced depressive phenotypes, such as dopamine or noradrenaline transporter knock-outs (Perona et al. 2008). To investigate the effect of selection on the monoamine signaling pathways we performed a series of pharmacological tests.

Although in a previous study we did not observe differences in VO_2swim across subsequent trials (Jaromin et al. 2016), here VO_2swim increased in the second trial of both of the pharmacological experiments, while activity time did not change (Supplementary Tables S1.5 and S1.7). In the third trial, VO_2swim was still higher than in the first trial, while activity time decreased. Those interesting results suggest that swimming in 38°C, that is, at temperature similar to the body temperature of voles, does not induce an immediate despair syndrome (a decrease of active behavior) in most individuals. The despair syndrome is connected with repeated stressful situation and is observed in rodents subjected to repeated forced swimming test (FST—about 5 min-long test performed in 23–25°C and widely used to investigate antidepressant drugs characteristics; Dal-Zotto et al. 2000; Cryan et al. 2005a). As ambient temperature is known to influence performance and the response to drugs (Roelands and Meeusen 2010), the results of our swimming trials and FSTs may not be directly compared.

All the drugs decreased VO_2swim (note that the order of treatments was randomized, so the reported effect of drugs is not due to the measurement order). However, in the case of vanoxerine (DARI) the result was opposite to that expected from studies on laboratory rodents. As vanoxerine rapidly increases the level of dopamine 30 min after injection, it usually increases physical activity in rats (e.g., Esumi et al. 2013). However, it did not affect behavior in some swimming tests with mice (Perona et al. 2008) or physical activity in humans (review in Roelands and Meeusen 2010). In high doses, vanoxerine can even decrease cardiac output (Knuepfer and Gan 1997), which is a trait closely related to VO_2. As bank voles are not used in standard pharmacological experiments, the data concerning their response to particular pharmaceuticals are scarce or lacking. As the response to drugs differs greatly between species and even strains (Cryan et al. 2005b; Petit-Demouliere et al. 2005), the sometimes unexpected results of the pharmacological trials can be specific for bank voles.

Fluoxetine (SSRI) decreased both VO_2swim and the proportion of activity time in the voles (Figures 4A,C,D and 5A,C,D). An opposite response to that drug could have been expected as SSRIs usually decrease or do not change immobility time in FSTs (see review Cryan et al. 2005a). However, as mentioned before, our swimming trials should not be directly compared with FSTs. Our results are consistent with the Central Fatigue Hypothesis, according to which the increased concentration of extracellular serotonin in several brain regions contributes to the induction of fatigue during prolonged exercise (Meeusen et al. 2006; note that the fatigue occurring as a consequence of physical exercise, that is, a physiologically "normal" situation, is a state principally different from pathological fatigue accompanying, for example, depression or chronic fatigue syndrome; although SSRI can accelerate the onset of fatigue during exercise, they can have a different effect in case of the pathological fatigue and are used in the treatment of that disease; Thomas and Smith 2006). Results from studies on animals suggest that pharmacological enhancement of serotoninergic signaling decreases physical performance. For instance, the injection of serotonin receptor agonist decreases the running time to exhaustion in rats (review in Roelands and Meeusen 2010) and SSRI decreases wheel running in mice (Rhodes et al. 2001; Weber et al. 2009). However, pharmacological manipulations of serotoninergic signaling in humans often do not influence the prolonged exercise (review in Meeusen and Roelands 2010). Thus, it was suggested that serotonin alone rather do not induce fatigue during prolonged exercise, but may play some role in combination with other neurotransmitter signaling (Meeusen and Roelands 2010). Similarly, the response to reboxetine (NERI) in voles was consistent with that reported in other studies concerning prolonged exercise, which generally show that selective NERIs decrease physical performance (e.g., Roelands et al. 2008; Weber et al. 2009; Klass et al. 2012; however, NERIs decrease immobility time during FSTs; Wong et al. 2000). That paradoxical effect (reboxetine increases the level of norepinephrine in a synaptic gap and norepinephrine is widely recognized as an activator) could be due to an activation of inhibitory α_2-autoreceptors that leads to a decrease in norepinephrinergic neurons firing (Mitchell et al. 2006). In voles, reboxetine decreased VO_2swim (Figure 4A), but did not change the proportion of activity time (Figure 4D). Apparently, VO_2swim is a more sensitive measurement of the exercise intensity than the simple behavioral observation. The results cannot be used to make inferences concerning the relative contributions of each monoaminergic system to the motivation, as the specific drugs may inhibit the reuptake transporters with different intensity (and moreover their dosages were arbitrary). After the drugs administration, we did not observe any suspicious symptoms, such as lack of a normal response to a stimuli (e.g., the attempt to catch), bristled fur, uncoordinated movements, trembling, etc. Thus, the observed decreased level of VO_2swim should be primarily due to modification of a particular monoamine signaling pathway rather than occurrence of an adverse event (e.g., anxiety that would decrease swimming and therefore the level of VO_2swim). As all the drugs influenced the intensity of "voluntary" exercise measured as the level of VO_2swim, the results confirm involvement of the monoamines in the motivation to be physically active. However, as no compounds are completely specific (i.e., the "specific" drugs still have some affinity to other neurotransmitter reuptake transporters and receptors), it is possible that the drugs decreased VO_2swim by modifying also other signaling pathways. Because monoamines regulate many behavioral states, we cannot exclude a possibility that the decreased VO_2swim was an effect of, for example, decreased arousal rather than decreased motivation. Thus, further studies are required to confirm that the monoamines affect the voles' activity by modifying the "motivation" *per se*.

To investigate the effect of selection on monoamine signaling pathways, we tested line Type × Drug interactions in additive models and line Type effect in the proportional response analyses. The

interactions in additive models were not significant, except for the analysis of the time of achieving VO_2swim. Specifically, in C lines the time of achieving VO_2swim tended to be accelerated after NERI, while in A lines it tended to be delayed after SSRI and NERI injections (Figure 4B). However, post hoc saline versus drug comparisons performed for A and C lines separately were not significant. Nevertheless, that differential response may indicate differences in serotonin and norepinephrine signaling between A and C lines. The proportional response to DARI in high dose, NERI, and SSRI was stronger in C lines than in A lines, but the difference was significant only in the case of NERI (Figures 4F and 5F). The result suggests that norepinephrine signaling pathway is involved in the mechanism responsible for differences in behavior between A and C lines, but further studies are required to confirm the role of norepinephrine in the evolution of increased aerobic exercise performance. In another selection experiment with mice, administration of SSRI decreased wheel running both in mice selected for high wheel running and unselected, control mice (Rhodes et al. 2001). However, HPLC analysis of brain tissue indicated lower serotonin level in selected mice when compared with unselected ones (Waters et al. 2013). The selected mice respond differently to serotonin 5-HT_{1A} receptor agonist and antagonist than control mice (Claghorn et al. 2016). Thus, possibly, usage of different methods (e.g., HPLC) or drugs in future studies will allow better detection of potential differences in monoamines concentration or signaling pathway in the brains of voles from A and C lines.

It could be argued that the lack of clear differences between the selected and control lines in response to the drugs resulted from the fact that the effects of the drugs were generally small, and that the effects could be stronger if higher doses were applied. However, in the case of too high a dose (i.e., overdose) the peripheral side-effects, for example, tachycardia, are more likely to occur (e.g., Borys et al. 1992; Whiskey and Taylor 2013), whereas the objective of this work was to investigate the central monoaminergic effects of the drugs. Moreover, a relevant pharmacological manipulation applied to test hypotheses concerning mechanisms underlying evolution should not lead to effects grossly exceeding the normal range of behavioral plasticity. Note also that we compensated for the (expected) small effect size by performing the tests on a large number of animals.

The knowledge concerning neurobiological basis of behavior is severely biased by results from experiments conducted on a few model species (Keifer and Summers 2016). Our results suggest the involvement of dopamine, serotonin, and norepinephrine in the regulation of motivation to be physically active in a non-model species of rodent. Moreover, the result suggests that evolution of increased physical performance in bank voles can be associated with changes in norepinephrine signaling. However, as the pharmacological approach has several limitations (e.g., not complete specificity of the drugs and their systemic application), the conclusions require an additional support. As the selection experiment is continued, further studies, for example, analyses of monoamine content in brain tissue or molecular analyses of the expression and allelic differences in genes coding monoamine receptors or involved in monoamine regulation (cf. Konczal et al. 2015, 2016), can be undertaken to resolve the question about the role of these monoamines in the evolution of increased aerobic exercise performance.

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Supplementary Material

Supplementary material can be found at http://www.cz.oxfordjournals.org/.

Conflict of Interest statement

The authors declare no conflict of interest.

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